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Mesoscopic organic nanosheets peeled from stacked 2D covalent frameworks†

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Novel mesoscopic organic nanosheets were developed by functionalizing bulk 2D organic covalent framework polymers with small molecules. The water-soluble fluorescent nanosheets are promising as nanocarriers for biological applications.

Nanosheets, which possess nanoscale features in the *x*-*y* plane, and single or limited atomic layers in the *z* axis, have emerged as important nanostructured materials due to their unique properties and potential applications.¹ Current studies of nanosheets are focused on inorganic materials (*e.g.* metal oxides and metal sulfides) and graphene-based materials.^{2–5} Two-dimensional (2D) polymer sheets are of great interest, however, the synthesis of free-standing polymeric sheets remains a major challenge, especially of mesoscopic (5–500 nm) organic nanosheets.^{6–9} In addition, functionalization of nanosheet materials for specific application is of great importance.

Porous organic frameworks have been created *via* the cross-coupling of rigid monomers.^{10–14} 2D covalent organic frameworks can spontaneously assemble to form macroscopic three-dimensional (3D) porous polymers.¹³ Therefore, it is critical to prevent this assembly process to obtain 2D organic nanosheets. Although a recent study attempted to create a thin layer of an organic framework on a solid surface,¹⁵ the synthesis of free-standing nanosheets by the separation of one 2D framework from another has yet to be achieved due to the strong interlayer forces. In general, 2D covalent frameworks are synthesized *via* the condensation or coupling of different rigid monomers with certain functional groups.^{16–18} Based on retrosynthetic thinking, the covalent framework can be split or functionalized by reaction with small molecules, especially for those frameworks linked by C–O or C–N bonds. Theoretically, it should be possible to peel away a thin layer of 2D covalent framework from a stacked 3D system to

generate novel functionalized organic nanosheets. Using this hypothesis, we have devised a unique synthesis of free-standing mesoscopic organic nanosheets.

Recently, we have synthesized a novel microporous polyisocyanurate (**PICU-A**) material by the cyclotrimerization of 3,3'-dimethoxy-4,4'-biphenylene diisocyanate (**A**) over an *N*-heterocyclic carbene (NHC) catalyst.¹⁹ **PICU** was built from a 2D covalent framework. Due to the irreversible reaction involved, **PICU** is an amorphous material with many terminal groups.¹⁹ These defects in the stacked layered structure made **PICU** a good candidate for functionalization to derive nano-sheets. In general, defects or terminal groups were more active towards reaction with small molecules. To derive different organic nanosheets, fluorene-based **PICU-B** was synthesized (Scheme 1).

Firstly, 3-aminopropanol was reacted with the terminal isocyanate (–NCO) group of **PICU** (Scheme 1). Typically, 10 mg of **PICU-A** and 0.2 mmol of 3-aminopropanol were added to a reaction vial containing 10 mL of *N,N'*-dimethylformamide (DMF). The vial was capped and heated at 100 °C for 24–72 h with stirring. The **PICU-A** suspension was converted to a brown solution (Figs 1A and B). After filtration, the **NS-Aa** obtained was precipitated and washed with diethyl ether.

The **NS-Aa** solid could be re-dissolved in methanol or DMF. However, it was nearly insoluble in water. To improve the solubility of the nanosheets in water, *o*-(2-aminoethyl)polyethylene glycol (PEG–NH₂) and D-glucosamine were used to synthesize nanosheets **NS-Ab** and **NS-Ac**, respectively. Similarly, **NS-Ba** and **NS-Bb** were prepared from **PICU-B**. **NS-Ab** was slightly soluble in water (<20 µg mL^{–1}), and the solubility of **NS-Bb** in water was even lower. In contrast, the glucosamine1-modified nanosheets **NS-Ac** showed good solubility in water (up to 2 mg mL^{–1}) (Fig. 1C).

The transmission electron microscopy (TEM) image shows the well-defined nanosheets of **NS-Aa**, **NS-Ba** and **NS-Bb** (Fig. 2). The size of the 3-aminopropanol-functionalized **NS-Aa1** nanosheets (reacted at 100 °C for 24 h) was in the range 70–150 nm (Fig. 2b), while the size of **NS-Aa2** nanosheet (reacted at 100 °C for 48 h) was only 40–80 nm (Fig. 2c). The **NS-Aa3** nanosheet (reacted at 100 °C for 72 h) was even smaller in size and irregular in shape (Fig. 2d). The nanosheets of 3-aminopropanol-functionalized **NS-Ba1** and PEG-functionalized **NS-Bb1** (both reacted at 100 °C for 24 h) are shown in Figs 2f and g, respectively. Fig. 2h shows that the nanosheets are amorphous, as is their polymer precursor. The nanosheets were derived from the reaction between the modification reagent R'–NH₂ and the terminal isocyanate (–NCO) group of **PICU** (Scheme 1). Given the short-range order in **PICU**, there were plenty of active terminal groups in the stacked layered structure. As a sufficient amount of modification reagents was attached to a piece of layered polymer, the surface layer(s) could be peeled off from the bulk polymeric particles to form nanosheets. The nanosheets could further react with R'–NH₂, and be peeled into thinner sheets or split into smaller pieces over a longer reaction period (Fig. 2). The atomic force microscopy (AFM) image (Fig. 3) also confirmed the nanosheet structure. Typically, the thickness of the nanosheets was ≤ 3 nm.

NS-Aa and **NS-Ba** showed very weak absorption in the visible range (Fig. S5A, ESI†). **NS-Ac** exhibited a blue fluorescence emission peak at 470 nm in water when excited at 365 nm (Fig.

1D), while **NS-Ba** only showed a very weak emission peak at 430 nm (Fig. S5B, ESI[†]). The blue fluorescent **NS-Ac** nanosheets could be used as a novel bioimaging agent. The application of water-soluble **NS-Ac** in biological systems was examined by confocal laser scanning microscopy (CLSM). After KB (human nasopharyngeal epidermal carcinoma) cells and RAW 264.7 (mouse leukemic monocyte macrophage) cells were incubated in a phosphate buffered saline (PBS) solution (pH = 7) with 30 $\mu\text{g mL}^{-1}$ of **NS-Ac** at 25 °C for 24 h, a blue luminescence was observed in the cytoplasm of these living cells (Fig. S6, ESI[†]), demonstrating the use of organic nanosheets in bioimaging applications.

Inspired by the aforementioned study, we further examined the possibility of using **NS-Ac** nanosheets as nanocarriers for delivering hydrophobic molecules into living cells. 7-diethyl-amino-3,4-benzophenoxazine-2-one (Nile red) was used as a hydrophobic probe in this test. RAW 264.7 cells were incubated in a PBS solution (pH = 7) with an aqueous solution of Nile red/**NS-Ac** or with a control (aqueous solution of Nile red) at 25 °C for 6 h. CLSM images showed a bright red luminescence in the cytoplasm of the live RAW 264.7 cells incubated with Nile red/**NS-Ac** (Fig. 4a). In contrast, there was negligible luminescence in the RAW 264.7 cells incubated with Nile red only (Fig. 4b). This result clearly demonstrated that the **NS-Ac** nanosheets acted as carriers for the Nile red molecules and delivered them into the live cells. This was made possible by the non-covalent van der Waals interactions between the organic nanosheets and the guest hydrophobic molecules.^{4,20}

We assessed the cytotoxicity of **NS-Ac** on HepG2 and KB cells using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based assay. The nanosheets exhibited a very low cytotoxicity ($\text{IC}_{50} \gg 100 \mu\text{g mL}^{-1}$) in HepG2 (human liver carcinoma) and KB cells (Figs 5A and B). 6.25–100 $\mu\text{g mL}^{-1}$ of **NS-Ac** showed a limited inhibitory effect on the proliferation of HepG2 cells cultured in Dulbecco's Modified Eagle's Medium (DMEM), and of KB cells cultured in Roswell Park Memorial Institute (RPMI) 1640. At a high concentration of 100 $\mu\text{g mL}^{-1}$, **NS-Ac** only inhibited (i) HepG2 cell proliferation in 48 h by 1.5% with statistical significance, and (ii) KB cell proliferation in 48 h by 5.5% with statistical significance. In addition, **NS-Ac** was stable in water for at least 4 months.

In summary, novel mesoscopic (30–150 nm) organic nanosheets have been synthesized by functionalization of bulk polymeric materials with a 2D organic covalent framework. As glucosamine was used in the synthesis, water-soluble fluorescent nanosheets were achieved. The water-soluble organic nanosheets showed promising biological applications, for example, as nanocarriers to deliver hydrophobic molecules into living cells. The 2D structure, versatile functionalization and tunable size of these organic nanosheets are of interest for various applications. These unique materials are also attractive due to their low cost and ease of synthesis.

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List of Schemes

Scheme 1 The synthesis of the PICU polymer and organic nanosheets.

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- Fig. 1 Photographs of (A) **PICU-A** suspended in DMF, (B) a DMF solution of nanosheets **NS-Aa**, and (C) an aqueous solution of nanosheets **NS-Ac**. (D) A photograph of an aqueous solution of **NS-Ac** under excitation at 365 nm.
- Fig. 2 TEM images of (a) **PICU-A**, the nanosheets of (b) **NS-Aa1**, (c) **NS-Aa2**, (d) **NS-Aa3**, (e) **PICU-B**, (f) **NS-Ba1** and (g) **NS-Bb1**. (h) The electron diffraction pattern of **NS-Aa2**.
- Fig. 3 (A,B) AFM images of the nanosheets of **NS-Aa2** on the silicon dioxide substrate. (C) The height profile of the nanosheet marked with a dashed line in (B).
- Fig. 4 CLSM images of live RAW 264.7 cells incubated with (a) Nile red/**NS-Ac** and (b) Nile red at 25 °C for 6 h.
- Fig. 5 The cytotoxicity of **NS-Ac**. (A) HepG2 cells were seeded at a density of 5000 cells per well in a 96-well plate and cultured for 24 h in DMEM. (B) KB cells were seeded at a density of 5000 cells per well in a 96-well plate and cultured for 24 h in RPMI 1640. Next, **NS-Ac** of various concentrations ($0\text{--}100\text{ }\mu\text{g mL}^{-1}$) in PBS was added in both cases and, after 48 h, the cells were assayed for proliferation. A TECAN Safire2 Multifunctional Microplate Reader was used for the profiling of the absorbance at a wavelength of 555 nm. The signal was normalized against vehicle treatment ($0\text{ }\mu\text{g mL}^{-1}$ of **NS-Ac**). Data were obtained from six independent experiments and presented as the mean and standard error of the mean (SEM).

Scheme 1

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5